

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
7 September 2001 (07.09.2001)

PCT

(10) International Publication Number  
**WO 01/64110 A1**

- (51) International Patent Classification<sup>7</sup>: A61B 10/00, [US/US]; 9295 E. Karen Drive, Scottsdale, AZ 85260 A61K 31/54 (US).
- (21) International Application Number: PCT/US00/05387 (74) Agent: DRUMMOND, William, H.; Drummond & Duckworth, Suite 500, 4590 MacArthur Boulevard, Newport Beach, CA 92660 (US).
- (22) International Filing Date: 28 February 2000 (28.02.2000)
- (25) Filing Language: English (81) Designated States (national): AU, BR, CA, CN, CZ, HU, IL, IN, JP, KR, MX, NO, NZ, PL, RO, SG, SK, TR, UA, US, ZA.
- (26) Publication Language: English (84) Designated States (regional): Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
- (71) Applicant (for all designated States except US): ZILA, INC. [US/US]; 5227 North 7th Street, Phoenix, AZ 85014-2800 (US).
- (72) Inventors; and  
(75) Inventors/Applicants (for US only): BERNAL, Samuel, D. [US/US]; 20000 Calvert Street, Woodland Hills, CA 91367 (US). BURKETT, Douglas, D. [US/US]; 4736 E. Euclid, Phoenix, AZ 85044 (US). GREEN, Ralph, E.
- Published:  
— with international search report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 01/64110 A1

(54) Title: METHOD FOR DETECTING AND KILLING EPITHELIAL CANCER CELLS

(57) Abstract: A diagnostic method for detecting cancerous epithelial cells by selectively marking the mitochondria of the cancer cells, by delivering to the epithelium a cationic supravital mitochondrial marking agent. Selective killing of cancerous epithelial cells in the presence of normal cells is effected by delivering a cationic supravital mitochondrial marking agent to epithelial cancer cells. The killing agent can also comprise the reaction product of the marking agent and a cancer chemotherapeutic drug.

-1-

METHOD FOR DETECTING AND KILLING  
EPITHELIAL CANCER CELLS

Field of the Invention

This invention relates to methods for detecting epithelial cancer.

In another respect the invention pertains to methods for selectively killing epithelial cancer cells.

In a further aspect, the invention concerns methods for detecting epithelial cancer cells in the presence of normal cells and/or for selectively killing such cells, in which the mitochondria of cancer cells retains a mitochondrial marking agent for a time sufficient to permit identification and/or killing such cells.

Definitions

As used herein, the following terms have the indicated meanings:

//

//

-2-

"Cancer" or "cancerous" cells are used in the broad sense, to include invasive cancer cells, cancer-in-situ cells and severely dysplastic cells.

"Mitochondrial marking agent" means a compound that is selectively taken up by the mitochondria of living cancer cells and is selectively retained in cancer cells for a time sufficient to permit identification and/or killing or incapacitation thereof.

"Killing" of cells means either causing cell death or cell changes that render a cell incapable of reproduction and metastasizing.

"Adduct" means the reaction product of a mitochondrial marking agent and a cancer chemotherapeutic agent.

#### Background of the Invention

In-vivo diagnostic procedures for detecting malignant and premalignant epithelial lesions or carcinomas, employing dye compositions that selectively

-3-

"color" tissues that are abnormal due to dysplasia, hyperplasia, tumorigenesis and other active surface lesions, are known in the art. These diagnostic methods employ a dye that imparts color to a cancerous substrate, which is then detectable under light at visible wavelengths or a fluorescent dye that imparts color to the substrate, which is then detectable when illuminated by light at wavelengths outside the visible spectrum.

For example, procedures employing fluorescein and fluorescein derivatives are disclosed in Chenz, Chinese Journal of Stomatology (27:44-47 (1992)) and Filurin (Stomatologiia (Russian) 72:44-47 (1993)). These procedures involve application of the dye, followed by examination under ultraviolet light to detect the cancerous/precancerous tissue, which is selectively fluorescent. Another prior art procedure involves rinsing the epithelium with toluidine blue, followed by normal visual examination to detect any selectively stained tissue. Such procedures are disclosed, for example in the patents to Tucci et al. (U.S. Patent 5,372,801) and Mashberg (U.S. Patent 4,321,251). Use of certain other thiazine dyes and oxazine dyes in an analogous manner is disclosed in U.S. Patent 5,882,627

-4-

to Pomerantz.

Heretofore, it was theorized that such dyes selectively "marked" cancerous tissue because it was retained in the relatively larger interstitial spaces between the cells of cancerous tissue and would not efficiently penetrate the normally tight intracellular junctions of normal tissue or be selectively retained in such relatively smaller spaces.

Contrary to the belief that toluidine blue selectively marks cancerous epithelial tissue because it is selectively retained in the relatively larger interstitial spaces between cancer cells, the mechanism of such selective staining of epithelial tissue by cationic dyes, e.g., dyes such as rhodamine, fluoresceins, oxazine and thiazine dyes (including toluidine blue) and other cationic supravital marking agents is the selective uptake and selective retention of the agent in the mitochondria of cancer cells. In turn, this selective staining of and retention in the mitochondria is apparently due to the higher electrical potential (negative charge on the inside of the membrane) of cancerous mitochondrial cells as compared to normal

-5-

cells. See, e.g., Chen et al., Cancer Cells 1/The Transformed Phenotype, 75-85 (Cold Spring Harbor Laboratory, 1984); Lampidis, et al., Cancer Research 43, 716-720 (1983).

In fact, the selective marking and retention of the mitochondria of cancer cells by supravital cationic dyes and other supravital cationic marking agents are related to one of the very characteristics of cancer cells that appears to be responsible for their rapid cloning growth and metastasizing ability, namely, that the higher electrical potential of the mitochondria of cancer cells is the source of cellular energy and is the driving force for ATP (adenosine triphosphate) product of the cells.

#### Summary of the Invention

I have now discovered a method for *in-vivo* detection of cancerous epithelial cells by selective marking of the mitochondria thereof. My method comprises the steps of delivering to tissue in the locus of a suspect cancerous site on the epithelium (which contains both normal and cancerous cells), with a cationic supravital mitochondrial marking agent other than rhodamine, causing

-6-

said agent to be taken up and selectively retained in the mitochondria of cancer cells. The cancerous cells are then detectable by any suitable method, for example, instrumental or visual examination under visible light or under light of selected invisible wavelengths.

In a further embodiment, after the marking agent is taken up by the mitochondria, a rinse reagent is applied to the locus of the suspect cancerous site, thus enhancing the rate of release of the agent from the mitochondria of the normal cells and further increasing the selectivity of the diagnostic method.

According to another embodiment of the invention, I provide a method for selectively killing cancerous epithelial cells comprising the step of contacting cancerous cells in the locus of a suspect cancerous site with a cationic supravital mitochondrial marking agent, to cause cell death or to render the cancer cells substantially incapable of multiplication. The marking agent can be delivered to the cancer cells in a single discrete dose, or continuously, or in repeated discrete doses, with or without employing a rinse reagent after each dose.

-7-

In a further embodiment of the invention, I provide a method of improving the selectivity of cancer chemotherapeutic agents comprising the steps of forming an reaction product of a cationic supravital agent and a chemotherapeutic agent and delivering the reaction product to cancerous epithelial cells.

These, other and further embodiments of the invention will be apparent to those skilled in the art and a better understanding of the invention will be obtained from the following examples which are provided to illustrate the invention and not as indications of the scope thereof, which is defined only by the appended claims.

In the following working examples, cationic supravital mitochondrial marking agents, including

dyes, including toluidine blue O, alcian blue, malachite green, phenosafranin, acriflavine, pyronine Y, toluylene blue and brilliant green;

and



-8-

"non-dye" compounds, including peonidin, oxythiamine, tiemonium iodide, elliptinium acetate and furazolium chloride.

In order to be selectively absorbed and retained in cancer cell mitochondria, the marking agent or reaction product of marking agent + chemotherapeutic agent, must have a molecular weight of below about 5,000.

#### Example 1

##### Uptake and Retention of Mitochondrial Marking Agents in Living Carcinoma Cells

Different concentrations of the various cationic marking agents, at 100, 50, 10 and 1  $\mu\text{g/ml}$  are prepared in RPMI medium complete with 20% fetal calf serum, 1 mM glutamine, hydrocortisone, insulin, transferrin, estradiol, selenium and growth hormone.

The carcinoma cells are incubated at 37°C in tissue culture incubators with 5%  $\text{CO}_2$  and 95% relative humidity, for 5 minutes with each agent and concentration there and then rinsed twice using 2 minute incubations with 1% acetic acid. After incubation and rinsing, the cells are

-9-

harvested, at 30 min., 1 hour, 2 hours, 4 hours and 8 hours. The cells are then extracted with 2-butanol and analyzed by spectrophotometry for quantitation of the marking agent.

The results show that there is a concentration dependence in the rate of accumulation of marking agent in the mitochondria of both carcinoma and normal cells and in the selectivity of release of the marking agent from cancer cells, but this concentration dependence starts to become less pronounced. The saturation concentration for toluidine blue O occurs at concentrations of 10µg/ml and above. The saturation concentrations for the other marking agents are similarly determined. The remaining experiments are conducted with a concentration of 10µg/ml for toluidine blue O and at the saturation concentrations for the other marking agents so-determined, unless stated otherwise.

#### Example 2

##### Mitochondrial Localization of the Agents in Living Cells

After incubation and rinsing of various cell lines, using the different cationic marking agents, the mitochondrial localization of the agents is analyzed

-10-

using confocal high resolution microscopy and phase contrast microscopy.

Living cells, are cultivated in complete growth medium with 20% fetal calf serum and growth factors, and maintained at 37°C. These cells accumulate and retain the marking agents in the mitochondria. When these cells are then maintained in a agent-free medium, carcinoma cells retain the agent for longer than about 1 hour, but normal epithelial cells release the agent within about 15 minutes.

In contrast to living cells, dead cells or cells treated with agents that dissipate the mitochondrial membrane potential lose mitochondrial staining and accumulate the agents in the nucleus.

### Example 3

#### Release of the Agents from Mitochondria With Dissipation of the Mitochondrial Membrane Potential

Known agents that alter the mitochondrial electrical potential are used to pretreat epithelial cancer cells, followed by treatment with the cationic supravital

-11-

mitochondrial marking agents. These pretreatment agents include azide and cyanide preparations and dinitrophenol.

Epithelial cancer cells are also pre-stained with the various dyes and then are post-treated with these known agents. The release of the dyes from the cells or the transfer of the dyes to other subcellular compartments, including the nucleus is analyzed.

The cells pretreated with these agents did not accumulate dyes in the mitochondria and the mitochondria of the pre-stained cells released the dye upon post-treatment with these agents.

Example 4

Tissue Explants of Squamous Carcinomas

Fresh explants of resected epithelial carcinomas are analyzed for marking agent uptake and retention. After resection, the carcinomas are microdissected from surrounding tissue, cut into 3 mm sections and maintained as explant tissue cultures at 37°C. These explants are then incubated with the various agents and then extracted for quantitation of the agent.

-12-

Oral carcinoma (SqCHN) have rapid uptake and prolonged retention of these agents. The agents start to be released from the cells after about one hour of cultivation in agent-free medium. However, the agents are released faster when the cells are incubated in medium that does not contain growth factors, fetal calf serum and other medium additives. The agents are also released faster when the cells are grown in adverse conditions such as lower temperatures.

#### Example 5

##### Tissue Explants of Normal Epithelial Cells

Cells obtained surgically from normal areas of the oral epithelium are cultivated as normal epithelial cultures. These cultures are then incubated with the marking agents for analysis of the agent uptake and retention.

Unlike the carcinoma cells, normal epithelial cells quickly release the agents from their mitochondria and from the cell much more quickly. By 10-15 minutes, most of the agent is released from the mitochondria.

-13-

**Example 6****Marking Agent-Chemotherapeutic Agent Adducts**

In place of the agents of Examples 1-5, the following adducts of cationic mitochondrial marking agents and various known chemotherapeutic agents are employed, with substantially similar results, except that the cancer cell kill rate and selectivity of the chemotherapeutic agent substantially improved.

**Marking Agent**

toluidine blue O

rhodamine 123

**Chemotherapeutic Agent**

methotrexate

nitrogen mustard

-14-

CLAIMS:

Having described my invention in such manner as to enable those skilled in the art to understand and practice it and, having identified the presently preferred embodiments thereof, I CLAIM:

-15-

1. A diagnostic method for *in vivo* detection of cancerous epithelial cells by selective marking of the mitochondria thereof, comprising the step of delivering to the epithelium a cationic supravital mitochondrial marking agent.

2. A method for selective killing of epithelial cancer cells comprising the step of delivering to epithelial cancer cells a cationic supravital mitochondrial marking agent.

3. The method of claim 2 in which the agent is the reaction product of a cationic supravital mitochondrial marking agent and a cancer chemotherapeutic drug.



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/05387

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(7) :A61B 10/00; A61K 31/54 US CL :424/9.6; 514/222.2 According to International Patent Classification (IPC) or to both national classification and IPC														
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 424/9.6, 9.7, 9.8; 514/222.2, 226.2, 226.8, 227.5; 258; 435/371; 436/64, 800, 813 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) APS, CAPLUS, MEDLINE, EMBASE search terms: epithelial, mitochondrial, carcinoma, cancer, toluidine blue, rhodamine, chemotherapy, imaging														
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>														
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.												
X	US 5,372,801 A (MALMROS et al.) 13 December 1994, see columns 1-2.	1												
Y	US 4,816,395 A (HANCOCK et al.) 28 March 1989, see columns 2-4.	3												
X	BERNAL, S. et al. Anticarcinoma activity in vivo of rhodamine 123, a mitochondrial-specific dye. Science. October 1983, Vol. 222, pages 169-172, entire document.	1-2												
Y		3												
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.														
<table border="0"> <tr> <td>* Special categories of cited documents.</td> <td>* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>*A* document defining the general state of the art which is not considered to be of particular relevance</td> <td>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>*E* earlier document published on or after the international filing date</td> <td>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>*L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>*A* document member of the same patent family</td> </tr> <tr> <td>*O* document referring to an oral disclosure, use, exhibition or other means</td> <td></td> </tr> <tr> <td>*P* document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </table>			* Special categories of cited documents.	* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	*L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family	*O* document referring to an oral disclosure, use, exhibition or other means		*P* document published prior to the international filing date but later than the priority date claimed	
* Special categories of cited documents.	* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention													
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone													
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art													
*L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family													
*O* document referring to an oral disclosure, use, exhibition or other means														
*P* document published prior to the international filing date but later than the priority date claimed														
Date of the actual completion of the international search 09 MAY 2000		Date of mailing of the international search report <b>13 JUN 2000</b>												
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer MICHAEL G. HARTLEY Telephone No. (703) 308-1235 JOYCE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATRIX 												